Autocrine Stimulation of Interleukin-1α and Transforming Growth Factorα Production in Human Keratinocytes and Its Antagonism by Glucocorticoids

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Interleukin-1 (IL-1) and transforming growth factorα (TGFα) mRNA expression was analyzed in cultured normal human keratinocytes. Keratinocytes constititively express IL-1 mRNA when cultured in keratinocyte growth medium but not in Dulbecco's minimal essential medium containing fetal bovine serum, in which the cells differentiate. The predominant form of IL-1 expressed by keratinocytes is IL-1α. Addition of IL-1α to keratinocytes increased IL-1α and TGFα mRNA expression in a dose-dependent manner. TGFα induced a similar increase in IL-1α and TGFα mRNA in keratinocytes. Hydrocortisone decreased the expression of both IL-1α and TGFα mRNA in keratinocytes. These findings document an autocrine mechanism by which IL-1α and TGFα can stimulate the proliferation of keratinocytes in the skin. It is proposed that this autocrine loop may be hyperactive in psoriasis. Antagonism of the effects of this autocrine loop may be one of the mechanisms by which glucocorticoids exert clinically useful effects in psoriasis and other diseases of the skin. *J Invest Dermatol 97:106–110, 1991*

The skin represents the largest organ of the body, and one frequently involved in both the induction phase and the effector phase of immune responses. In the seventies Streilein suggested the concept of skin-associated lymphoid tissue (SALT) [1]. In his model, Langerhans cells (LC) were thought to present antigens to skin-associated lymphocytes. More recently it has been shown that keratinocytes, which comprise the great majority of cells in the epidermis, produce several cytokines, including IL-1, IL-3, IL-4, and IL-6 [2–5]. Interferon-γ (IFNγ) induces in keratinocytes the expression of class II MHC glycoproteins [6], as well as intercellular adhesion molecules (ICAM), which can mediate binding and retention of subsets of T-lymphocytes [7]. Keratinocytes produce IL-1, which can act as a co-factor for T-lymphocyte proliferation [2]. Thus keratinocytes as well as LC may function as antigen-presenting cells to T-lymphocytes.

Cytokines produced by keratinocytes are also of interest in diseases characterized by increased proliferation and decreased differentiation of these cells, e.g., psoriasis [8]. Keratinocytes in psoriatic lesions express increased levels of IL-1α mRNA [9]. Moreover, transforming growth factorα (TGFα), a potent stimulator of keratinocyte proliferation, is overexpressed in keratinocytes in psoriatic lesions [10,11]. Addition of TGFα to cultured keratinocytes increases TGFα mRNA levels, suggesting autoinduction of this cytokine [10]. The question arose whether the overexpression of IL-1α and TGFα in psoriatic keratinocytes were independent events or whether they might be causally related. To investigate this possibility, IL-1α and TGFα were added to keratinocytes in culture and levels of IL-1α and TGF-α mRNA were analyzed. Each cytokine was found to induce not only its own mRNA but also that of the other cytokine. This suggested the existence of an autocrine loop involving both cytokines and linked to keratinocyte proliferation. An inhibitor of this autocrine loop was then studied. Hydrocortisone was found to decrease the inductive effects of both IL-1α and TGFα, as well as antagonizing the induction by IFNγ of HLA-DR expression. Differentiation of keratinocytes was found to restrict IL-1α production.

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Abbreviations:

BPE: bovine pituitary extract
CHX: cycloheximide
DMEM: Dulbecco's minimum essential medium
FCS: fetal calf serum
HC: hydrocortisone
ICAM: intracellular adhesion molecules
IFNγ: interferon gamma
IL-1α: interleukin-1 alpha
IL-1β: interleukin-1 beta
KBM: keratinocyte basal medium
KGM: keratinocyte growth medium
LC: Langerhans cells
LPS: lipopolysaccharide
MHC: major histocompatibility complex
PBS: phosphate-buffered saline
PMA: phorbol 12-myristate 13-acetate
SALT: skin-associated lymphoid tissue
TGFα: transforming growth factor alpha
TFGβ: transforming growth factor beta
TNFα: tumor necrosis factor alpha

MATERIALS AND METHODS

Isolation and Culture of Human Keratinocytes Keratinocytes were prepared from adult fresh human skin according to a previously described method [12] or purchased from Clonetics Corporation, San Diego, CA. Keratinocyte fractions depleted of Langerhans cells (LC) were prepared by panning with the monoclonal antibody CD1 (Becton Dickinson, Mountain View, CA) as described previously [13]. Keratinocytes (3×10^4) were cultured rou-

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tinely in 10-cm² tissue culture dishes with defined keratinocyte growth medium (KGM, Clonetics Corporation) at 37°C in 5% CO_2. Five to seven days after seeding, keratinocytes were washed and the medium was replaced with KGM, KGM without hydrocortisone, or keratinocyte basal medium (KBM, see figure legends). Alternatively, keratinocytes were grown in Dulbecco's minimum essential medium (DMEM, GIBCO, Grand Island, NY) plus 10% fetal calf serum (FCS) and antibiotics as described previously [12]. Keratinocytes from both sources behaved similarly in responses evaluated.

Extraction and Analysis of RNA in Keratinocytes Cells were treated as indicated in the figure legends. Cell lysates were dot blotted according to procedures described by White and Bancroft [15]. Alternatively, cells were washed in KBM and dissolved in a solution of RNAzol (Cinna/Biotecx, Friendswood, TX) containing guanidine isothiocyanate and phenol [18]. One-tenth volume of chloroform was added and the mixture was incubated at 4°C for at least 15 min. Total RNA was isolated from the aqueous phase of this extraction by centrifugation at 10,000 × *g* and precipitated with 1 volume of isopropanol. The RNA pellet was washed in 70% ethanol, dried, and heat-denatured in 7.5% formaldehyde and 6 × SSC (20 × SSC = 3 M NaCl, 0.3 M Na citrate) at 60°C for 15 min and rapidly cooled at 4°C. RNA was blotted onto Nytran paper (Schleicher and Schuell Inc., Keene, NH). Blots were baked for 30–120 min at 80°C in a vacuum chamber.

Prehybridization and hybridization mixtures were composed of 5× Denhardt's solution (0.1% BSA, 0.1% Ficoll, Sigma Chemical Co., St. Louis, MO), 5 × PIPES (0.75% M NaCl, 25 mM piperazine-N,N¹-bis-2-ethane-sulfonic acid (PIPES, Sigma) and 0.25 mM EDTA), 50% formamide, 0.2% SDS, 50 mM Na phosphate, 100 μg/ml yeast tRNA (Sigma), 100 μg/ml salmon sperm DNA (Sigma). Hybridization was carried out at 42°C in a water bath for 4–24 h. cDNA probes were labeled with ^{32}P by nick-translation (BRL, Rockville, MD) to a specific activity of at least 10^8 cpm/μg. Blots were hybridized with at least 10^6 cpm/ml for 17–24 h, washed for two 1-h periods at 42°C in 0.2× SSC/0.2% SDS, 1 h at 65°C in 0.2× SSC/0.2% SDS, and exposed at −70°C on Kodak XAR films (Eastman Kodak Co., Rochester, NY) with an intensifying screen. The extent of hybridization of cDNA to RNA was quantitated by scanning the films with an optical densitometer (Hoefer Scientific Co., San Francisco, CA). Data was collected using a Macintosh computer with a data acquisition software (Dynamax, Rainin Instrument Co., Woburn, MA), normalized to β-actin mRNA levels and expressed as relative mRNA levels.

cDNA, Cytokines, and Chemicals Human IL-1α and IL-1β, TGFα, and β-actin cDNA were kindly provided by Immunex Corporation (Seattle, WA), R. Derynck (Genentech, South San Francisco, CA), and B. Endlich (University of California, San Francisco, CA), respectively. HLA-DR cDNA was purchased from American Type Culture Collection (Rockville, MD). IL-1, TNFα, TGFα, and IL-6 were obtained from Immunex Corporation, Cetus Corporation (Emeryville, CA), Genentech, and P. Sehgal (Rockefeller University, New York, NY), respectively. EGF, IFNγ, and rat TGFα were purchased from Clonetics, Amgen (Thousand Oaks, CA) and Peninsula Labs (Belmont, CA), respectively. Lipopolysaccharide (LPS, *E. coli* 0111:B4), cycloheximide (CHX), and phorbol 12-myristate 13-acetate (PMA) were purchased from Sigma.

IL-1 Assays Culture supernatants were collected and IL-1α and IL-1β were assayed in a two-site sandwich ELISA as described by Kenney et al [16,17].

RESULTS

Human Keratinocytes Express IL-1 mRNA Keratinocytes cultured in KGM 7 d after seeding expressed IL-1α mRNA (Fig 1). Lipopolysaccharide (LPS) did not affect the level of IL-1α mRNA. A slight increase of IL-1α transcript was observed when cycloheximide (CHX) was added to the confluent cultures. When keratinocytes were grown in DMEM plus FCS, no mRNA for IL-1α could be demonstrated. Incubation with LPS or CHX did not change this pattern of expression (Fig 1). The absence of IL-1 mRNA expression by keratinocytes cultured in high CA^{++}-containing DMEM (1.8 mM) suggests that IL-1 mRNA expression may be restricted by cell differentiation. This is consistent with the finding of Ansel et al that murine keratinocytes induced to differentiate terminally by growth in high-Ca^{++} medium (1.2 mM) ceased to express IL-1 mRNA [18].

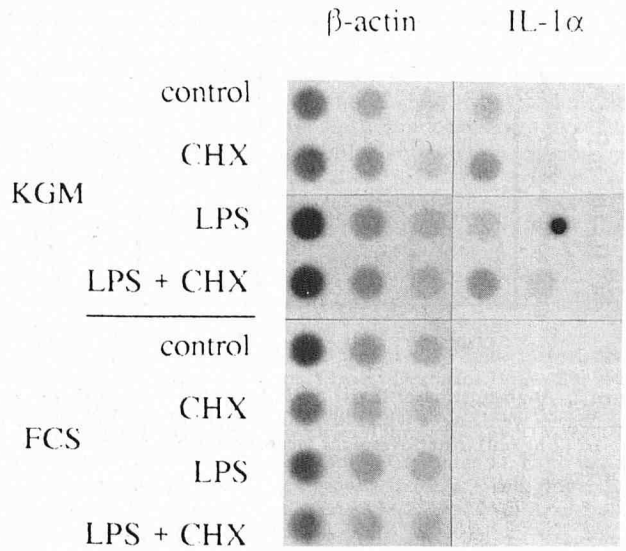

Figure 1. Expression of IL-1 mRNA in keratinocytes. Subconfluent keratinocytes cultured in KGM or DMEM with 10% FCS were treated with LPS (20 μg/ml). After 19 h of incubation, CHX (10 μg/ml) was added and further incubated for 2 h. Threefold dilutions of cell lysates were prepared and dot-blotted onto Nytran paper. *Left panel,* β-actin probe; *right panel,* IL-1α probe. The spot overlapping partially the second dilution of LPS-stimulated keratinocytes cultured in KGM is an artifact.

Effect of IL-1α on IL-1α and TGFα mRNA Although both IL-1α and β mRNA were detected in keratinocytes (data not shown), the mRNA for IL-1α was the predominant species. IL-1β was barely detectable in culture supernatants by immunoassay (Table I), so that the alpha form of IL-1 was subsequently used in all studies, and only IL-1α mRNA expression was analyzed. To determine whether IL-1α mRNA could be induced by exogenous stimuli, we added IL-1α (2 ng/ml) and other known IL-1 inducers to keratinocyte cultures and analyzed the mRNA 24 h later. IL-1α slightly increased the level of its own mRNA (Fig 2). However, PMA (100 nM) stimulated IL-1α mRNA to a greater extent. In contrast, LPS and PMA co-stimulation, a combination that induced an elevated and sustained IL-1 mRNA response in promonocyte cell line U937 [19], did not increase the IL-1 mRNA expression above

Table I. Expression of IL-1α and IL-1β in Supernatants of Keratinocyte Cultures[a]

	Day 1		Day 2		Day 3	
Culture Medium	IL-1α	IL-1β	IL-1α	IL-1β	IL-1α	IL-1β
KGM	15	NT	16	<11	22	<11
KGM minus HC	ND	ND	208	<11	43	<11

[a] Subconfluent keratinocyte cultures were washed three times with KGM without HC. After 3 h of incubation, the medium was removed and replaced with fresh KGM or KGM without HC. Supernatants were collected at selected intervals and analyzed by an immunoassay. Sensitivity of the assay is about 10 pg/ml and variability is less than 5%. NT, not tested; ND, not done.

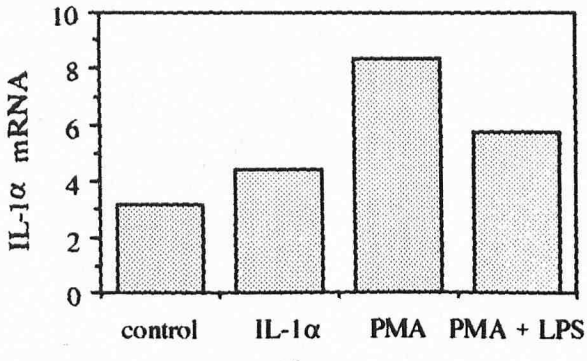

Figure 2. Augmentation of IL-1α mRNA expression by PMA and IL-1. Keratinocytes were treated with PMA (100 nM), PMA, and LPS (20 μg/ml) or IL-1α (2 ng/ml). After overnight incubation, RNA were extracted and analyzed.

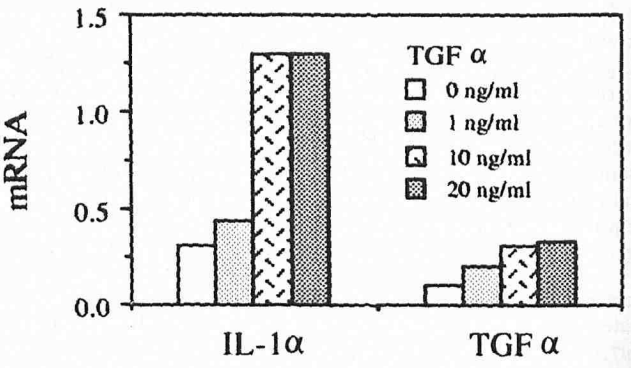

Figure 4. Induction of IL-1α and TGFα mRNA by TGFα treatment. Before stimulation, keratinocyte cultures (Clonetics) were washed three times with KBM. After 3 h of incubation in KBM, fresh KBM was added to the cultures and cells were treated with TGFα (1, 10, or 20 ng/ml, Genentech) and analyzed after overnight incubation.

that following PMA treatment alone. LPS by itself had no effect (Fig 1).

A supplement in KGM is hydrocortisone (HC, 0.5 μg/ml). Glucocorticoids have been shown to reduce IL-1 mRNA accumulation in cells of monocytic lineage [19], therefore HC was removed from KGM before the cells were stimulated. We analyzed the effects of IL-1α on IL-1α and TGFα mRNA accumulation following IL-1α treatment in the presence or absence of hydrocortisone. In accordance with results from previous experiments (Fig 2), IL-1α stimulated synthesis of its own mRNA (Fig 3). In the absence of HC, the stimulation by 2 ng/ml IL-1α was almost twofold over that in the presence of HC. With higher IL-1α doses, the difference between cultures with and without HC was less marked. Thus, HC partially decreases IL-1α mRNA expression in keratinocytes. In addition, IL-1α augmented TGFα mRNA accumulation, and this response was also reduced by HC. This experiment suggests that IL-1α may modulate proliferation of keratinocytes through expression and auto-induction of TGFα, and that glucocorticoids can antagonize this effect. Further investigations are required to confirm the translation of TGFα mRNA and secretion of this cytokine.

The Effect of TGFα on Synthesis of IL-1α and TGFα mRNA Keratinocyte growth medium (KGM) contains several other supplements, including bovine pituitary extract (BPE, 1 : 250) and epidermal growth factor (EGF, 10 ng/ml). BPE has been reported to be a rich source for TGFα [20]. Keratinocytes grown in complete KGM may thus be subjected to modulation by TGFα,

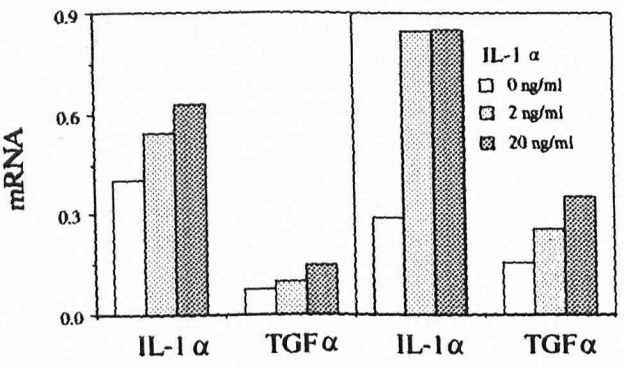

Figure 3. Effects of IL-1α and HC on IL-1α and TGFα mRNA expression. Before stimulation, keratinocytes (Clonetics) were washed three times with KGM without HC. After 3 h of incubation, medium of keratinocyte cultures was removed and replaced with fresh KGM or KGM without HC. Several concentrations of IL-1α were added to the cultures and RNA were extracted and analyzed after overnight incubation. *Left panel,* KGM; *right panel,* KGM without HC.

because TGFα and EGF have been shown to be important for sustained growth of keratinocytes [21], and TGFα can upregulate its own mRNA [11]. EGF alone has been reported to enhance the proliferation of keratinocytes [22,23]. Both TGFα and EGF bind to the same EGF receptors and trigger expression of TGFα mRNA and protein [10]. Whether TGFα has any effect on IL-1α expression had not been investigated. To understand the complex interactions of cytokines and glucocorticoids it is necessary to remove these supplements. Keratinocytes were washed three times with KBM. After 3 h of incubation in KBM, the medium was replaced with fresh KBM and various amounts of TGFα. The absence of BPE and EGF did not abrogate IL-1α mRNA expression (Fig 4). In accordance with a previous report [11], TGFα stimulates its own mRNA expression. Moreover, IL-1α mRNA was also augmented by TGFα treatment in a dose-related manner. Addition of BPE to keratinocytes likewise caused a slight increase of IL-1α mRNA expression (data not shown), an observation consistent with the report that TGFα is present in BPE [20].

The Effects of Various Cytokines on HLA-DR mRNA Induced by IFNγ Because IFNγ induces the mRNA for HLA-DR in human keratinocytes [6] and IL-1 antagonizes the class II MHC-inducing effect of IFNγ in several cell types, including monocytes, normal skin fibroblasts, and chondrosarcoma and astrocytoma cell line [24], we investigated the effect of IL-1α and other cytokines (IL-6, EGF, TNFα, and TGFα) on IFNγ-induced HLA-DR mRNA expression (Fig 5). None of these cytokines except IFNγ (500 U/ml) increased HLA-DR mRNA (data not shown). Both TNFα and TGFα consistently decreased HLA-DR mRNA expression induced by IFNγ in the presence or absence of HC. The deletion of HC from KGM caused a slight but consistent increase of HLA-DR mRNA. HLA-DR mRNA was also increased when IL-1α or IL-6 was added with IFNγ. EGF had no demonstrable effect in modulating HLA-DR mRNA expression. Whereas this result is preliminary, it shows that the class II MHC mRNA induced by IFNγ can be modulated differentially by TGFα and IL-1α, both of which are produced in proliferating keratinocytes.

DISCUSSION

The first point to emerge from our studies is that the predominant form of IL-1 produced by keratinocytes is IL-1α, in contrast to monocytes, where the predominant form is IL-1β [25]. In keratinocytes cultured by a different method Kupper et al [4] likewise found a predominance of IL-1α. The second conclusion is that whereas IL-1α is expressed when keratinocytes are cultured in KGM, this cytokine mRNA is not detectable when the cells are cultured in DMEM, which contains 1.8 mM Ca^{++}. These observations, and

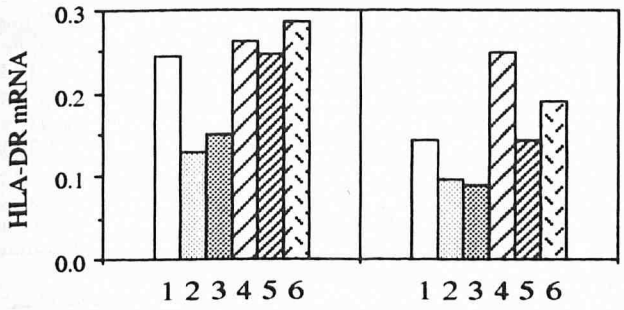

Figure 5. Effect of interferon-γ and cytokine cotreatment on HLA-DR mRNA expression. Medium of keratinocyte cultures was removed and replaced with fresh KGM *(right panel)* or KGM without HC *(left panel)*. IFNγ (500 U/ml) and cytokines were added to all cultures for 48 h. *1,* control; *2,* TGFα (10 ng/ml, Peninsula Lab); *3,* TNFα (20 ng/ml); *4,* IL-1α (2 ng/ml); *5,* EGF (100 ng/ml); *6,* IL-6 (150 U/ml).

report that when murine keratinocytes terminally differentiate by growth in 1.2 mM Ca^{++} medium they cease to express IL-1 mRNA [18], suggest that IL-1α gene expression may be restricted to the conditions when keratinocytes are proliferating. Terminally differentiated alveolar macrophages likewise produce much less IL-1β than monocytes cultured and stimulated under comparable conditions [26]. If these observations can be extrapolated to the in vivo situation, IL-1α production would be confined to the less differentiated basal keratinocytes in the epidermis.

IL-1α and TGFα mRNA are constitutively expressed by keratinocytes cultured in KGM, and addition of either IL-1α or TFGα can augment the levels of both these cytokines mRNA. This raises the possibility that IL-1α and TGFα can in vivo amplify their own production by an autocrine mechanism. Because TGFα is a potent stimulator of keratinocyte proliferation [21], and both IL-1α and TGFα are produced in psoriatic epidermis [9,11], hyperactivity of the autocrine loop just described may play a pathogenetic role in psoriasis. Antagonism of this autocrine stimulation by glucocorticoids may contribute to their clinical efficacy in that disease.

Although Langerhans cells (LC) are believed to be the principal antigen-presenting cells in the epidermis [27], keratinocytes may also function as antigen-presenting cells [2]. IFNγ induces in keratinocytes the expression of class II major histocompatibility glycoproteins [6,7] and intercellular adhesion molecules (ICAM), which mediate the binding and retention of subsets of T-lymphocytes [8]. The only additional factor required for antigen presentation of T-lymphocytes is a co-stimulator. The IL-1α in keratinocytes may function in this way, as evidence is accumulating that membrane-associated IL-1α can act as a co-stimulator in the induction by human peripheral blood β-lymphocytes and monocytes of the proliferation of T-lymphocytes [28]. Antigen presentation by keratinocytes could contribute to resistance against infections, for example, those produced by herpesviruses, bacteria, and fungi. Activation of T-lymphocytes in such infections would release IFNγ, which induces HLA-DR and ICAM expression in keratinocytes, thereby maintaining their antigen-presenting capacity. Inhibition by glucocorticoids of IFNγ and IL-1α–induced HLA-DR mRNA expression, as now reported, could contribute to the immunosuppressive and anti-inflammatory effects of these compounds in the skin. The hypothesis that glucocorticoids induce the production of lipocortins, proteins inhibiting phospholipase A_2 activity, is not supported by recent evidence [29].

An interesting possibility is that keratinocytes and LC present antigens to different subsets of T-lymphocytes. Activated keratinocytes do this in the skin itself, whereas LC-carrying antigens can also migrate through afferent lymphatics to the paracortical areas of lymph nodes of the drainage chain [30]. In this location they are termed interdigitating cells, the dendritic extensions of which are in close opposition to T-lymphocytes [30]. In contrast to keratinocytes, which produce little IL-1β, stimulated LC produce approximately equal amounts of IL-1α and IL-1β.† Contact-sensitizing chemicals bound to LC very efficiently induce delayed hypersensitivity [31], so this is a situation in which antigen presentation by LC may predominate.

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